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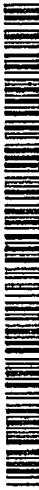
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(54) Title: PROCESS FOR FINDING A PROTEASE INHIBITOR

(57) Abstract: The present invention relates to a process for binding substances capable of specifically inhibiting membrane-bound proteases and a high throughput screening test for finding substances capable of specifically inhibiting γ-secretase or presenilinase. The invention further relates to the use of this process or this high throughput screening test for finding substances which are specifically capable of inhibiting γ-secretase or presenilinase. It also discloses substances which can be found with a process according to the invention, the use of said substances according to the invention for preparing a medicament for treating neurodegenerative disorders, particularly Alzheimer's disease and pharmaceutical formulations containing the substances according to the invention.

Process for finding a protease inhibitor

The present invention is in the field of processes for
finding inhibitors of proteases membrane-based; these are
5   in particular γ-secretase and presenilinase inhibitors.
Another embodiment of the invention relates to the use of
these inhibitors for preparing a medicament for the
treatment of neurodegenerative diseases as well as
pharmaceutical formulations which contain these
10   substances. The aggregation and precipitation of
proteins are involved in the origins of various
neurodegenerative diseases such as Alzheimer's,
Parkinson's and Huntington's chorea. In Alzheimer's
disease the amyloid-β-peptide (Aβ) aggregates and leads
15   to insoluble senile plaques which constitute one of the
pathological markers of the disease (Selkoe et al.,
1996). Aβ is formed by the proteolytic cleaving of a
precursor protein, the amyloid precursor protein (APP).
Two metabolic routes of APP have been distinguished, the
20   non-amyloidogenic route and the amyloidogenic route
(Selkoe, 1994).
In the non-amyloidogenic metabolism of APP, α-secretase
cleaves within the Aβ region of the APP and thus leads to
the secretion of the soluble N-terminal region of the
25   protein (α-APP) and after the cutting of γ-secretase
leads to the release of p3. By contrast, the
amyloidogenic route leads to the formation of Aβ, as two
proteases generate the N-terminus (β-secretase) or
C-terminus (γ-secretase) of Aβ (Haass, 1993; Selkoe,
30   1994).
In vivo, Aβ can be detected in human plasma and
cerebrospinal fluid. Secreted Aβ can also be detected in
cell culture, in the cell culture supernatant of various
cell types which endogenously express or overexpress APP
35   or fragments thereof. Both Aβ production and hence the
formation of amyloid plaques are influenced by various

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genetic risk factors. These include mutations in the
homologous proteins presenilin 1 and presenilin 2 and in
APP itself. The analysis of these mutations on
fibroblasts from Alzheimer's patients with familiar
5   Alzheimer's disease (FAD) demonstrated the influence
which they have have on Aβ production. This was
confirmed by investigations on transfected cells and
transgenic animals. All mutations increase the
production of Aβ and, in the case of the presenilin
10 mutations, lead to a selective increase in the longer Aβ
variant, Aβ42 (Selkoe, 1996; Price, 1998). This peptide
aggregates to a greater extent than the shorter form,
Aβ40, and has a more toxic effect on cells of neuronal
origin than on those of peripheral origin (Lemere et al.,
15 1996; Mann et al., 1996). In addition to this effect of
the mutations, there are indications that the Wild type
form of the presenilins also have a fundamental function
in the physiological formation of Aβ. In neurones from
mouse embryos in which the PS1-gene (PS: presenilin) had
20 been switched off by genetic engineering, there was found
to be a drastic reduction in Aβ40 and Aβ42. Moreover,
the C-terminal fragments of APP accumulated in the cells,
leading to the view that the presenilins activate
γ-secretase or themselves have γ-secretase activity (De
25 Strooper et al., 1998; Sisodia et al., 1998). Early in
vitro test systems combined with mutation studies on
conserved aspartates of presenilin 1 led one to assume
that the presenilins could be special autocatalytically
active aspartate proteases which are responsible for the
30 γ-secretase in the membrane (Wolfe et al., 1999).
The discussion of the identification of γ-secretase as
the decisive step in the generation of Aβ and hence in
the origins of Alzheimer's has still not concluded.
Quite independently, this protease constitutes an
35 interesting target for intervening pharmacologically in
the process of Aβ formation by finding inhibitors which

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selectively reduce the activity thereof. It is important
to develop not only animal models and in vitro test
systems but also cellular assays which will make it
possible to test specific active substances irrespective
5   of transporting processes within the cell.
Wolfe et al. (1999) have disclosed an in vitro system for
measuring the activity of γ-secretase. In order to
prepare the system, membranes from cells which express
PS1 in a stable manner are worked up. They are mixed
10 with a plasmid coding for the LC99-polypeptide and
subjected to an in vitro transcription/translation
reaction in the presence of ^{35}S-methionine, to form the
γ-secretase substrate C99. The mixture is then incubated
under the appropriate conditions, during which time the
15 C99 fragment is proteolytically cleaved from APP by the
γ-secretase and the breakdown products are detected by
gel electrophoresis after immunoprecipitation. This test
system is very time-consuming and incapable of being
automated and is not therefore suitable for finding
20 specific protease inhibitors.
At the moment, there are no known therapeutic approaches
for preventing neuronal cell death on the basis of
presenilin fragments which appear to be involved in
neuronal apoptosis. Presenilinase, i.e. the protease
25 activity which generates said presenilin fragments, is an
excellent target molecule. No methods are known from the
prior art for finding presenilinase inhibitors,
particularly not in an industrial context.
The aim of the invention is to provide an improved test
30 system for finding specific protease inhibitors. This
aim can be achieved by means of the present invention
within the scope of the specification and claims.

Before the present invention is described in more detail,
35 it should be pointed out that all the plurals also
include the singulars and vice versa, i.e. if a reference

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is made to "the substances" individual substances are
also covered by the invention.

Brief description of the present invention

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The present invention relates to a process for finding
substances capable of specifically inhibiting membrane-
based proteases, and a high throughput sampling test for
finding substances which are capable of specifically

10   inhibiting γ-secretase or presenilinase. The invention
further relates to the use of this process or this high
throughput sampling test for finding substances which are
able to inhibit specifically γ-secretase or
presenilinase. The invention also discloses substances

15   which can be found with a process according to the
invention, the use of said substances according to the
invention for preparing a medicament for treating
neurodegenerative diseases, particularly Alzheimer's
disease, and pharmaceutical formulations which contain

20   the substances according to the invention.

Details of the Figures

Figure 1: Preparation of reporter constructs and cell
25   lines.

Figure 2: Carrying out the test system ⚝ HTS assay
principle.

30   Figure 3: ER retention of the γ-secretase substrate ⚝
intracellular APP processing.

Figure 4: Characterisation of the transfected cell lines
for HTS.
35   A) Determining the Aβ-KKK/Gal4 substrate expression
by Western blot.

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B) Determining the luciferase expression by Western blot.

C) Determining the luciferase expression by enzymatic detection.

D) Determining the luciferase expression by enzymatic detection.

Figure 5: Determining the levels of extracellular Aβ42 secreted by the transfected HTS test cell lines.

Figure 6: BFA effect in the HTS test cell lines Aβ-KKK-ER/59 and Aβ-KKK/52.

A) Luciferase test.

B) Vitality test.

Figure 7: Effect of presenilin (PS1 and PS2) aspartate mutations in the HTS test cell lines Aβ-KKK-ER/59 and Aβ-KKK/52

A) Effect of the PS (Asp) mutants in the pool cells Aβ-KKK/52/DN385N (PS1mut) and Aβ-KKK/52/D366A (PS2mut).

B) Effect of the PS (Asp) mutants in the pool cells Aβ-KKK-ER/59/D385N (PS1mut) and Aβ-KKK-ER/59/D366A (PS2mut).

C) Detection of overexpression of the PS (Asp) mutants in the particular cell lines.

Figure 8: Substance A.

A) Concentration-dependent inhibition of Aβ formation by substance A.

B) Concentration-dependent inhibition of Aβ 40 and Aβ 42 secretion by substance A.

Figure 9: Substance B.

Concentration dependent inhibition of Aβ formation by substance B.

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<u>Figure 10:</u> Substance C.
Concentration dependent inhibition of Aβ formation by
substance C.

5   Detailed description

According to the invention, a process is provided for
finding substances capable of specifically inhibiting
proteases which cleave substrates membrane-based, which
10   is characterised in that
        a) cells are cultivated which have said protease
           activity endogenously or exogenously and express
           a membrane-associated recombinant fusion protein
           which comprises the substrate of said protease
15   with the specific cleavage site for said protease
           and a reporter,
        b) these cells are incubated with a test substance,
        c) the quantity of reporter cleaved is measured and
        d) the value obtained is compared with the value
20   obtained in the absence of the test substance.
     By said protease of protease activity is meant an enzyme
     or an enzymatic activity which cleaves specifically, i.e.
     between certain amino acid sequences, and does not cleave
     between other amino acid sequences, and the substrates of
25   which are membrane-based or are membrane-associated. By
     membrane-based is meant, within the scope of this
     invention, that the substrate is an integral part of the
     membrane. By membrane associated, for the purposes of
     the invention, is meant that the substrate is bound to
30   the surface of the membrane or to integral membrane
     proteins. This definition of membrane associated
     substrates should also include substrates which interact
     via chemical groupings with the hydrophobic part of the
     membrane which are added by posttranslational
35   modifications. Moreover, the term membrane associated
     substrates should also include substrates which interact

via amino acid side chains with the hydrophobic part of
the membrane, albeit to a lesser extent than the integral
membrane proteins. As an example, prostaglandin
synthetase may be mentioned here. By substrate is meant
5   peptide and proteins which contain at least one cleavage
site of said protease. The substrates may also be
modified, i.e. glycosylated for example (Sambrook et al.,
1989). According to the invention, said substrate is
fused to a reporter (see below) which serves to detect
10   the protease activity (cleaving). The fusion proteins
according to the invention may be produced by current
methods of genetic engineering (Sambrook et al., 1989) if
the DNA coding for the substrate and reporter protein is
available. The DNA which codes for the reporter proteins
15   may be obtained, for example, from commercial suppliers
such as Clontech of Heidelberg and inserted into the
desired vectors by standard procedures (Sambrook and
Maniatis, 1989). The DNA which codes for the substrate
can be obtained for proteases from suitable gene banks
20   using standard methods.
Test substances may be any substances known in the art or
new substances. For example, these substances may be
proteins or chemical compounds which are also available
in commercial libraries of substances. By comparing the
25   value obtained with the value obtained in the absence of
the test substance, as described in part d) of the
process according to the invention, the specificity of
the process according to the invention can be monitored.
With such monitoring the skilled person is able to
30   identify those substances which exhibit the inhibition of
said proteases according to the invention.
In particular, the process according to the invention is
characterised in that cells are cultivated which express
a fusion protein which contains the specific cleavage
35   site of γ-secretase (see also Example 1). The fusion
protein with the γ-secretase substrate according to the

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invention may be membrane associated in general terms but
is preferably membrane-based.
Moreover, the process according to the invention is
characterised in that cells are cultivated which express
5   a fusion protein containing the specific cleavage site of
presenilinase.
In a particular embodiment the process according to the
invention is also characterised in that the fusion
protein contains amyloid β. This is the substrate of
10   γ-secretase.
For a particular embodiment of the process according to
the invention, the fusion protein contains a fragment of
amyloid β. This represents the substrate for the
γ-secretase and according to the invention must contain
15   at least one specific γ-secretase cutting site.
For one particular embodiment of the process according to
the invention, the fusion protein contains the amyloid
precursor protein or a fragment thereof. For example,
the fusion protein may consist of a reporter protein and
20   the C99 fragment. The DNA which codes for the
γ-secretase substrate or for the C99 fragment can be
obtained from suitable gene banks using standard methods.
This constitutes the substrate for the γ-secretase and
according to the invention must contain at least one
25   specific γ-secretase cutting site.
For another particular embodiment of the process
according to the invention the fusion protein comprises
presenilin 1 or a fragment thereof. The fragments may
be, for example, N-terminal fragments (NTF) of about
30   21-28 kDa or C-terminal fragments (CTF) of 16-24 kDa
(Haas et al., 1998; Okochi et al., 1997; Thinakaran et
al., 1996), which must nevertheless go beyond the
presenilase cutting site. Presenilin 1 or the fragments
thereof constitute the substrates of the presenilinase
35   and according to the invention must contain at least 1
specific presenilinase cutting site.

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In another particular embodiment of the process according
to the invention, the fusion protein comprises presenilin
2 or a fragment thereof. The fragments may for example
be N-terminal fragments (NTF) of about 28-30 kDa (NTF)

5   kDa or C-terminal fragments (CTF) of 20-25 kDa (Haas et
al., 1998; Kim et al., J Biol Chem 1997, 272,
11006-11010; Podlisny et al., 1997), which must go beyond
the presenilinase cutting site. Presenilin 2 or the
fragments thereof constitute the substrates for the

10   presenilinase and according to the invention must contain
at least one specific presenilinase cutting site.
Preferably, the process according to the invention is a
high throughput screening $\times$ HTS test. This HTS has
advantages over the prior art since it allows a number of

15   substances to be tested for their effectiveness by a
simple method. A high throughput test or HTS is a method
of investigating a large to very large number of
substances simultaneously. Preferably, an HTS may be
carried out in microtitre plates, it may be partially or

20   totally automated and may be connected to electronic
equipment such as computers for data storage, analysis
and evaluation using bioinformatics. Preferably, this
automation makes use of both robots, as used in the
automobile industry, which are capable of handling large

25   numbers of microtitre plates simultaneously or
sequentially, and also automatic pipetting machines.
These automatic or semi-automatic HTS systems are capable
of performing several thousand tests a day and are
commercially available. Test substances can be acquired

30   commercially as libraries of substances. The test
substances are preferably dissolved in an organic solvent
and are most preferably dissolved in DMSO. Preferably,
the test substances which exhibit the desired protease
inhibitor function in a cell-free system are also tested

35   in a cell-based system. The term high throughput test or
HTS also includes ultra-high throughput screening: UHTS.

10

Preferably, these ultra-high throughput tests or UHTS are
carried out in 384- or 1536-well microtitre plates with
sub-microlitre or sub-nanolitre pipettors, improved plate
reading equipment and processes which prevent
5   evaporation. HTS methods are described for example in US
Patents 5876946 A or 5902732 A. The average person
skilled in the art will be aware of other literary
sources on HTS tests and can adapt the processes of the
present invention to an HTS or UHTS format without any
10 inventive step and without having to be inventive
themselves. Within the scope of this invention, a
reporter protein should be taken to mean a protein having
the capability of generating an easily detected signal
the quantity of which correlates with the quantity of the
15 cleavage product in question. The signal is generated
either by the measurement of the enzymatic activity of
the reporter protein with substances which are easily
detected or by measuring the intensity of fluorescence of
the reporter protein. Examples of reporter proteins are
20 the green fluorescent protein (GFP) (see for example
WO95/07463) or derivatives thereof which fluoresce at
other wavelengths or enzymes such as luciferase,
secretory alkaline phosphatase or β-galactosidase.
Therefore, according to another preferred embodiment of
25 the process according to the invention, the quantity of
reporter cleaved is measured with luciferase, green
fluorescent protein or a derivative thereof, secretory
alkaline phosphatase or β-galactosidase.
In yet another preferred embodiment of the process
30 according to the invention the quantity of reporter
cleaved is detected indirectly.
A large number of transcription factors are known, which
require two subunits for their activity. In other cases,
a single transcription factor may require two separate
35 functional domains for its activity (e.g. one
transcriptional activator domain and one DNA binding

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domain) so that each domain on its own is inactive but
when they are brought together the transcriptional
activity can take place.
One of these factors is Gal4 which, as described, can be
5   divided into two domains (Laughan, A and Gesteland, R
Molec. Cell Biol., 1984, 4: 260-267; Fields and Song,
Nature, 1989, 340: 245). Other transcription factors are
members of the Jun, Fos and ATF/CREB families, Oct1, Sp1,
HNF-3, the steroid receptor superfamily and others.
10 Similarly, in yet another preferred embodiment of process
according to the invention, the quantity of reporter
cleaved can be detected indirectly when a cleaved
reporter domain migrates into the nucleus, binds to
another reporter domain of a reporter construct therein
15 and the expression of the reporter is activated.
In yet another preferred embodiment of the process
according to the invention, the quantity of reporter Gal4
cleaved is detected indirectly when Gal4 in the nucleus
binds to the Gal4 DNA binding domain of another reporter
20 construct and the expression of luciferase is activated
(see Examples 2 and 4).
In yet another preferred embodiment of the process
according to the invention the quantity of reporter
cleaved is detected directly. For example, this might be
25 direct detection of the cleaving by the protease by means
of a reporter which is only activated when cleaving
occurs, for example. Examples of reporters may be those
described above: GFP, or enzymes such as luciferase,
secretory alkaline phosphatase or β-galactosidase.
30 The invention may be performed using any cells or cell
lines known in the art, particularly eukaryotic cells or
cell lines. Preferred cells or cell lines are those used
in neurological or neurobiological research, e.g.
mammalian cells or cell lines such as H4, U373, NT2,
35 HEK 293, PC12, COS, CHO, fibroblasts, myeloma cells,
neuroblastoma cells, hybridoma cells, oocytes, embryonic

stem cells.   It is also possible to use insect cell lines
(e.g. using Baculovirus vectors such as pPbac or pMbac
(Stratagene, La Jolla, CA)), yeast (e.g. using yeast
expression vectors such pYESHIS (Invitrogen, CA)) and
5   fungi.
Particularly preferred are cells or cell lines of
neuronal or glyol origin.   In a most particularly
preferred embodiment of the invention the cells used are
H4 cells, human neuroglioma cells from the brain which
10   were deposited under ATCC Number HTB-148 at the American
Type Culture Collection (ATCC) in Manassas, Virginia,
USA.
Therefore, in a special embodiment of the process
according to the invention, the cells are of neuronal or
15   glial origin.
In one particular embodiment of the process according to
the invention the cells are H4 cells.
According to the invention, a high throughput screening
test for finding substances capable of specifically
20   inhibiting γ-secretase is characterised in that
    a) H4 cells are cultivated which endogenously or
        exogenously have said γ-secretase activity and which
        express the membrane associated recombinant fusion
        protein Aβ-KKK/Gal4,
25   b) these cells are incubated with a test substance.
    c) the quantity of transactivator Gal4 cleaved and
        binding to the Gal4 DNA binding domain of the
        luciferase construct is measured by means of the level
        of luciferase expression and
30   d) the value obtained is compared with the value obtained
        in the absence of the test substance.
This high throughput screening test according to the
invention is described in more detail in the Examples.
The processes or high throughput screening tests
35   according to the invention may be used within the scope

of the invention to find substances capable of
specifically inhibiting γ-secretase.
The processes or high throughput screening tests
according to the invention may be used within the scope
5   of the invention to find substances capable of
specifically inhibiting presenilinase.
The invention also includes substances which can be found
using a process or high throughput screening test
according to the invention, characterised in that they
10   specifically inhibit the proteolytic cleaving of a
γ-secretase substrate. For example, these substances may
be proteins or chemical compounds. A substance according
to the invention is substance A as shown in the following
formula (see also Example 8):

15
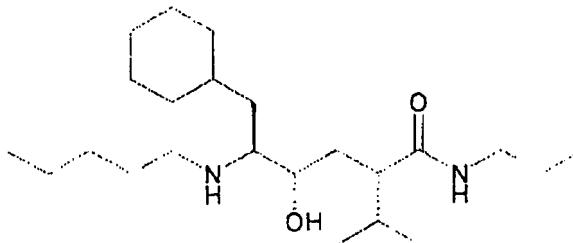
The invention further relates to substances which can be
found using a process or high throughput screening test
according to the invention, characterised in that they
specifically inhibit the proteolytic cleaving of a
20   presenilinase substrate. These substances may be
proteins or chemical compounds, for example. Said
substances according to the invention may be used within
the scope of this invention for preparing a medicament
for treating neurodegenerative diseases, particularly
25   Alzheimer's disease. Other neurodegenerative diseases
include for example Alzheimer's, Parkinson's and
Huntington's chorea. The invention also relates to a
pharmaceutical formulation which contains a substance

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according to the invention as well as conventional
pharmaceutical carriers.

A pharmaceutically acceptable carrier may contain
physiologically acceptable compounds which increase the
5   stability or absorption of the substance according to the
invention, for example. Such physiologically acceptable
compounds contain, for example, carbohydrates such as
glucose, saccharose or dextrans, antioxidants such as
ascorbate or glutathione, chelating agents, proteins of
10 low molecular weight or other stabilisers (cf. for
example Remington's Pharmaceutical Sciences (1990)).
Anyone skilled in the art knows that the choice of a
pharmaceutically acceptable carrier including a
physiologically acceptable compound depends on the route
15 of administration, for example.

The Examples which follow are intended to illustrate the
invention without restricting its scope in any way.

15

Example 1 § Preparation of reporter constructs and cell
lines (see also Figure 1)
Carrying out the test system:

5   1.1 Preparing a suitable cell line γ-secretase HTS:
     H4 neuroglioma cells (Accession Number HTB 148 at the
     American Type Culture Collection, Manassas, Virginia,
     USA) were stably transfected under standard conditions
     with the reporter construct pFRLuc (Stratagene) which
10   carries the gene for luciferase which is under the
     control of a promoter containing the Gal4 DNA binding
     domain. By transient transfection experiments with
     pcDNA3-Gal4 which codes for soluble Gal4 an individual
     clone was selected which showed the highest luciferase
15   activity. In order to prepare the Aβ-KKK/Gal4 construct
     a sequence which contained the N-terminal signal sequence
     of APP and the first 55 amino acids of Aβ (Shoji et al.,
     1992) was attached to the Gal4 coding sequence (Laughan,
     A and Gesteland, R Molec. Cell Biol., 1984, 4: 260-267)
20   by genetic engineering methods and cloned into the
     expression vector pcDNA3neo (Invitrogen). This construct
     was designated Aβ-KKK/Gal4. In order to insert an
     ER-retention signal in the substrate of the γ-secretase,
     the last nucleotides of the Gal4 were modified by genetic
25   engineering methods so that they coded for amino acids
     KKLI. This construct was named Aβ-KKK-ER/Gal4. The cell
     clone obtained as described above was used for the second
     stable transfection with pcDNA3/Aβ-KKK/Gal4 or
     pcDNA3/Aβ-KKK-ER/Gal4 and the selection was carried out
30   with neomycin under standard conditions (Sambrook and
     Maniatis, 1989). Individual neomycin-resistant cell
     clones were tested for expression of luciferase. The
     clones with the highest expression were used for the
     substance analyses. All the transfections were carried
35   out using the Fugene transfection system of Boehringer

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Mannheim in accordance with the manufacturer's
instructions.

1.2 Preparation of a suitable construct for presenilinase
5   HTS:
In order to identify inhibitors of presenilinase, a
construct was designed consisting of a substrate portion
(presenilin part) and a reporter part (Gal4). The
presenilin part consists of the nucleotides which code
10   for amino acids 3-101 and amino acids 263 to 325 of PS-1
(Sherrington et al., 1995). The first region contains
the first transmembrane region of PS-1. By gene
manipulation methods, the human APP signal sequence
(Shoji et al., 1992) was attached to the N-terminus of
15   the first presenilin sequence. The reporter fragment
adjoins the second presenilin region (Gal4, amino acid
1-881; Laughan, A and Gesteland, R Molec. Cell Biol.,
1984, 4: 260-267). This construct was cloned into the
expression vector pcDNA3neo (Invitrogen).
20

Example 2 ⅋ Carrying out the test system:
HTS Assay Principle (see also Figure 2).
The doubly stable HTS cell line is seeded onto a 96/384
microtitre plate. When the cells achieved confluence
25   they were incubated with the test substance in question
for a specified length of time. After incubation the
cell medium is removed and the luciferase enzyme activity
is determined precisely in accordance with the
instructions given by the manufacturer of the test kit
30   used (SteadyGlo, Promega or a different manufacturer).
By the presence of an endogenous or exogenous γ-secretase
activity in the H4 cells the substrate (Aβ-KKK/Gal4) is
cleaved proteolytically, whereupon the transactivator
Gal4 can detach itself from the membrane and enter the
35   cell nucleus. In the cell nucleus, Gal4 binds to the
Gal4 DNA binding domain of the reporter construct and

thus activates the expression of the luciferase. When a
specific γ-secretase inhibitor is present the substrate
cannot be cleaved and Gal4 remains bound to the substrate
on the cell membrane, leading to a reduction and even
5   total inhibition of the luciferase activity.

Cells are seeded onto 96/385 well plates in DMEM whole
medium (10% FCS, 1% glutamine, 1% penicillin/strepto-
mycin) in a dilution of 1:5. The cells are incubated for
10   24 to 48 hours (depending on the cell clone and dilution
used) at 37°C in 5% CO_2 and allowed to grow until 80-90%
confluence is achieved (which may take less or more
time). Then the test substance is added and incubated
overnight (8-16 hours) at 37°C and 5% CO_2. The 96/384
15   well plates are equilibrated at room temperature (RT).
The SteadyGlo Luciferase Assay System (Promega Catalogue
No.E2520) is used. The luciferase assay reagent is
thawed and equilibrated at ambient temperature or used
fresh (luciferase assay substrate dissolved in luciferase
20   assay buffer). The medium is suction filtered from the
cells. 100μl (based on 96 well plates) of fresh whole
medium are added to each well. 100μl (based on 96 well
plates) of luciferase assay reagent are added and
incubated for 5 minutes at RT. Then the luminescence is
25   measured. For 384 well plates the amounts pipetted are
reduced accordingly, in a manner known in the art.

Example 3: ER retention of the γ-secretase substrate &
intracellular APP processing (see Figure 3).
30   APP is a transmembrane protein which is processed along
the secretory metabolic route. The full length protein
is presumably already cleaved in very early cell
compartments such as ER (endoplasmatic reticulum) and
Golgi apparatus by the so-called β- and γ-secretases,
35   forming predominantly Aβ42 (Wild-Bode et al., 1997). In
the trans Golgi network (TGN), in the vessels tied off by

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the TGN and migrating to the cell membrane, or on the
cell membrane directly, the Aβ40 is presumably formed by
the activity of the same or similar proteases (Thinakaran
et al., 1996). APP which has not been processed by the
5   secretory metabolic route can enter the
endosomal/lysosomal system by endocytosis and there be
degraded to Aβ or degraded fully (Koo and Squazzo, 1994).
In order to increase the concentration of substrate in
those cellular compartments in which the formation of
10 Aβ42 takes place and in order to rule out the
identification of non-specific inhibitors such as
transporting inhibitors, an ER retention signal sequence
was appended to the γ-secretase substrate, the
Aβ-KKK/Gal4 fusion protein.

15

Example 4: Characterisation of the transfected cell lines
for HTS (see Figure 4).

A) Expression of the Aβ-KKK/Gal4 substrate.
20 A fairly large number of different doubly stable H4 cell
clones were prepared and investigated for the expression
of the Aβ-KKK/Gal4 substrate protein. Cell extracts were
prepared by standard methods, the proteins were separated
in a 10% SDS polyacrylamide gel and transferred onto a
25 PVDF membrane. The fusion protein was detected by
chemiluminescence with a primary antibody against Gal4
(Clontech; 1:5000) and with a secondary Horseradish
Peroxidase-coupled Antibody (Amersham; 1:6000).
By way of example, the expression of the fusion protein
30 is shown here in cell clones Aβ-KKK-ER/40, 59 and 65
(substrate Aβ-KKK/Gal4 with ER retention signal) and in
the cell clones Aβ-KKK/40 and 52 (substrate Aβ-KKK/Gal4
without ER retention signal). By contrast, no expression
could be detected in the untransfected H4 cells (u.t.)
35 and in the H4-Luc53 cells (luc53) which were transfected

only with a reporter construct Gal4dbd-Luc (Gal4-DNA-
Binding domain/luciferase; in English luciferase).

B) Determining luciferase expression by Western blot.

5   The cell clones Aβ-KKK-ER/40, 59 and 65 (substrate
Aβ-KKK/Gal4 with ER retention signal) and the cell clones
Aβ-KKK/40 and 52 (substrate Aβ-KKK/Gal4 without ER
retention signal) were investigated for the expression of
luciferase. Cell extracts were prepared by standard
10   methods, the proteins were separated in a 10% SDS
polyacrylamide gel and transferred onto a PVDF membrane.
The luciferase was detected by chemiluminescence with a
primary antibody (Europa) in a 1:100 dilution and with a
secondary horseradish peroxidase-coupled antibody
15   (Amersham; 1:6000).
The luciferase expression could be detected in all the
doubly stable cell clones but not in the untransfected H4
(u.t.) and the H4-Luc53 cells (luc53). As a positive
control, H4 cells were co-transfected with the various
20   substrates (soluble gal4 [sol. gal4], Aβ-KKK/Gal4 and
Aβ-KKK-ER/Gal4) and the reporter construct Gal4dbdLuc
(Gal4-DNA-Binding domain/luciferase; in English
luciferase). In these transient transfections, too, the
luciferase could be detected in the H4 cells.

25

**C) Determining luciferase expression by enzymatic
detection.**
The expression level of luciferase in the cell clones
Aβ-KKK-ER/40, 59 and 65 (substrate Aβ-KKK/Gal4 with ER
30   retention signal) and in the cell clones Aβ-KKK/40 and 52
(substrate Aβ-KKK/Gal4 without ER retention signal) was
also investigated by enzymatic activity measurement
(SteadyGlo, Promega). The luciferase assay was carried
out precisely according to the instructions of the
35   manufacturer (Promega).

Luciferase activity could be detected in all the doubly
stable cell clones but not in the untransfected H4 (H4)
and in the H4-Luc53 cells (luc53). In this test the
signal was not related to the amount of protein.

5

D) Determining the luciferase expression by enzymatic
detection.
The luciferase activity was determined in the two cell
clones Aβ-KKK-ER/59 (substrate Aβ-KKK/Gal4 with ER
10   retention signal) and Aβ-KKK/52 (substrate Aβ-KKK/Gal4
without ER retention signal). The luciferase assay
(SteadyGlo, Promega) was carried out exactly as
instructed by the manufacturer (Promega).
Luciferase activity could be detected in all the doubly
15   stable cell clones but not in the untransfected H4 (H4)
or in the H4-Luc53 (luc53) cells. In this experiment the
signal was related to the amount of protein. The
differences in signal intensity between the two cell
clones correlated with the expression level of the
20   substrate (see A).

Example 5: Determining the levels of extracellular Aβ40
and Aβ42 in the transfected HTS test cell lines (see
Figure 5).
25   The cell clones Aβ-KKK-ER/40, 59 and 65 (substrate
Aβ-KKK/Gal4 with ER retention signal) and the cell clones
Aβ-KKK/40 and 52 (substrate Aβ-KKK/Gal4 without ER
retention signal) were tested for their level of
extracellular Aβ40 and Aβ42. Fresh medium was added to
30   the confluent cells and these were incubated for a
further 16 hours. After incubation the medium was
removed and the concentration of Aβ40 and Aβ42 was
determined in a quantitative ELISA (Steiner et al.,
1998). Untransfected H4 cells were used as the control,
35   and the concentration of secreted Aβ40 and Aβ42 was put
at 100%.

21

The levels of extracellular Aβ42 in the cell clones
Aβ-KKK-ER/40, 59 and 65 were slightly raised, but no
difference could be detected for the quantity of secreted
Aβ40 in these clones. A higher concentration could not
5   be found for cell clone Aβ-KKK/40 and 52 either for Aβ40
or for Aβ42.

Example 6: BFA effect in the HTS test cell lines
Aβ-KKK-ER/59 and Aβ-KKK/52 (see Figure 6).

10   Cells of the clones Aβ-KKK-ER/59 (substrate Aβ-KKK/Gal4
with ER retention signal) and Aβ-KKK/52 (substrate
Aβ-KKK/Gal4 without ER retention signal) were treated for
6 hours with different concentrations of Brefeldin A
(BFA). Untreated cells were used as the control.

15   A) Luciferase test. The luciferase was detected by
enzymatic activity measurement. The luciferase assay was
carried out exactly as prescribed by the manufacturer
(SteadyGlo, Promega). A clear BFA effect could be seen
in cell clone Aβ-KKK/52 but not in cell clone

20   Aβ-KKK-ER/59. An increase in the BFA concentration leads
to an increased Aβ formation in the ER (Wild-Bode et al.,
1997) and hence to an increase in the luciferase signal.
B) Vitality test. A possible toxic effect of the BFA (in
different concentrations) on the cells was investigated

25   using an Alamar Blue test (see below) which was carried
out in parallel to the luciferase test. No loss of
vitality could be found over the entire range of
concentrations.

30   *Alamar Blue Measurement (Cytotoxicity measurement)*
Preparation:

Alamar Blue (Messrs. Biosource International, VE=100 ml,
Cat.No. DAL 1100 (AL-01-100)) is sterile-filtered. The
35   seeding of the cells and substance incubation are carried
out as in Example 1.

22

The Alamar blue incubation is carried out parallel to the
luciferase test.

5   Method:

Alamar blue is diluted to 5% with whole medium (DMEM m
4.5g/l glucose + 10% FCS + 1% glutamine + 1% Pen/Strep).
The medium is suction filtered. The cells are washed
10 once with medium or PBS. 100 µl of (5%) Alamar blue are
added (96 well plate) and a plate is incubated for 1 hour
in the incubator at 37° and in 5% CO_2. Fluorimetric
measurement is carried out at an input wavelength of
544 nm, an emission wavelength of 590 nm and a measuring
15 time of 0.3 s/well. The results are given as a
percentage of the control.

Example 7: Effect of presenilin (PS1 and PS2) aspartate
mutations in the HTS test cell lines Aβ-KKK-ER/59 and
20 Aβ-KKK/52 (see Figure 7).
The cell clones Aβ-KKK-ER/59 (substrate Aβ-KKK/Gal4 with
ER retention signal) and Aβ-KKK/52 (substrate Aβ-KKK/Gal4
without ER retention signal) were transfected with cDNAs
which coded for PS1 or PS2 and carried an aspartate(Asp)
25 mutation (PS1-D385N or PS2-D366A). 48 hours after
transfection the cells were selected with zeozin in order
to obtain pool cells.

A) Effect of PS (Asp) mutants in the pool cells
30 Aβ-KKK/52/D385N and Aβ-KKK/52/D366A.
The expression of the luciferase in the Aβ-KKK/52
(substrate Aβ-KKK/Gal4 without ER retention signal) pool
cells pool-PS1mut (D385N) and pool-PS2mut (D366A) was
investigated by enzymatic activity measurement. The
35 luciferase assay was carried out precisely in accordance
with the instructions of the manufacturer (SteadyGlo,

23

Promega). A significant reduction in the luciferase
signal to approximately 10% of the control value could be
detected in both pool cells. The H4-Luc53 cells were
used as a control.

5

B) Effect of the PS (Asp) mutants in the pool cells
Aβ-KKK-ER/59/D385N and Aβ-KKK-ER/52/D366A.
The expression of the luciferase in the Aβ-KKK-ER/59
(substrate Aβ-KKK/Gal4 without ER retention signal) pool
10 cells pool-PS1mut (D385N) and pool-PS2mut (D366A) was
investigated by enzymatic activity measurement. The
luciferase assay was carried out precisely in accordance
with the instructions of the manufacturer (SteadyGlo,
Promega). A reduction in the luciferase signal to ˜60%
15 (PS1mut), or ˜70% (PS2mut) of the control could be
detected in both pool cells. The H4-Luc53 cells were
used as a control.

C) Detecting the overexpression of PS (Asp) mutants in
20 the cell lines.
The pool cells Aβ-KKK/52/D385N and D366A and the pool
cells Aβ-KKK-ER/59/D385N and D366A were investigated for
overexpression of the presenilin aspartate mutant by
Western blot methods. Cell extracts were prepared by
25 standard methods, the proteins were separated in a 12%
SDS-polyacrylamide gel and transferred onto a PVDF
membrane. PS1 was detected with a polyclonal antibody
(5023; 1: 1000; Walter et al., 1997) and PS2 with a
monoclonal antibody (BI.HF5C; 1.3000; Steiner et al.,
30 1999b). As described (Steiner et al., 1999a; Wolfe et
al., 1999) both aspartate mutations prevent the
proteolysis of PS proteins so that only full length PS
can be detected. The overexpression of the exogenous PS
moreover leads in every case to a significant reduction
35 in the endogenous PS, which is apparent from the
reduction in PS-C-terminal fragments (CTFs).

24

Example 8: Substance A. (see Figure 8).

A) Concentration-dependent inhibition of Aβ formation by Substance A.

5   *Substance A:*
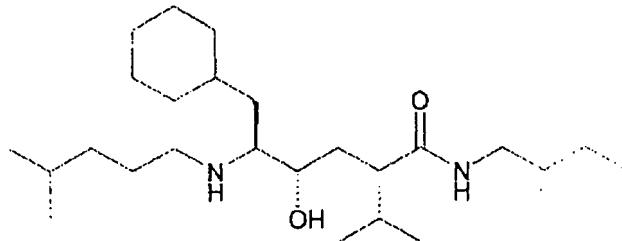
The cell clones Aβ-KKK-ER/40, 59 and 65 (substrate Aβ-KKK/Gal4 with ER retention signal) and the cell clone Aβ-KKK/52 (substance Aβ-KKK/Gal4 without ER retention

10 signal) was used to test the inhibitory effect of substance A. Confluent cells were incubated overnight with different concentrations of substance A. The luciferase assay was carried out exactly in accordance with the manufacturer's instructions (SteadyGlo,

15 Promega).

In all cell clones substance A inhibits Aβ formation in a manner dependent on concentration, as indicated by the decrease in the luciferase signal.

20 B) Concentration dependent inhibition of Aβ40 and Aβ42 secretion by substance A.

Substance A was also active in a cellular test system in which the extracellular content of Aβ40 and Aβ42 secreted by the U373 cell line (U373: ATCC No. HTB 14) was

25 determined. This cell line U373/APP751 is an astrocytoma cell line which overexpresses human APP751 and secretes large amounts of Aβ40 ($^\sim$1000pg/ml/4 hours with 5×10^7 cells in 15 ml of medium) and Aβ42 ($^\sim$100pg/ml/4 hours with 5×10^7 cells in 15 ml of medium). The measurement is

25

carried out by ELISA (Steiner et al., 1998). The Aβ40
secretion was greatly reduced in concentration-dependent
manner by substance A, whilst the Aβ42 secretion was
increased at subinhibitory doses and then also inhibited
5   at higher doses.

Example 9: Substance B (see Figure 9).
Effect of substance B on Aβ formation.
Substance B.

10
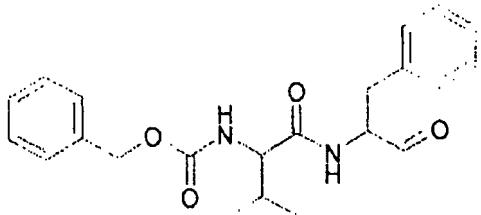
15

Cell clones Aβ-KKK-ER/40, 59 and 65 (substrate
Aβ-KKK/Gal4 with ER retention signal) and cell clones
Aβ-KKK/40 and 52 (substrate Aβ-KKK/Gal4 without ER
retention signal) were used to test the inhibitory effect
20 of substance B. Confluent cells were incubated overnight
with different concentrations of substance B. The
luciferase assay was carried out precisely in accordance
with the manufacturer's instructions (SteadyGlo,
Promega).
25 Substance B does not inhibit Aβ formation in
concentration-dependent manner in any of the cell clones,
as demonstrated by the constant signal intensity of the
luciferase.

2b

Example 10: Substance C (see Figure 10).
Effect of substance C on Aβ formation.

Substance C:

5

10
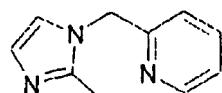
Cell clones Aβ-KKK-ER/40, 59 and 65 (substrate Aβ-KKK
with ER retention signal) and cell clone Aβ-KKK/52
(substrate Aβ-KKK without ER retention signal) were used
to test the inhibitory effect of substance C. Confluent
15 cells were incubated overnight with different
concentrations of substance A. The luciferase assay was
carried out precisely in accordance with the
manufacturer's instructions (SteadyGlo, Promega).
Substance C does not inhibit Aβ formation in
20 concentration-dependent manner in any of the cell clones,
as demonstrated by the constant signal intensity of the
luciferase.

27

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2*8*

Claims:

1. Process for finding substances which are capable of specifically inhibiting proteases which cleave membrane-
5 bound substrates, characterised in that
   a) cells are cultivated which exhibit said protease activity and express a membrane-associated recombinant fusion protein which comprises the substrate of said protease with the specific cleavage
10   site for said protease and a reporter,
   b) these cells are incubated with a test substance,
   c) the quantity of reporter cleaved is measured and
   d) the value obtained is compared with the value obtained in the absence of the test substance.
15 2. Process according to claim 1, characterised in that cells are cultivated which express a fusion protein which comprises the specific cleavage site of γ-secretase.
   3. Process according to claim 1, characterised in that cells are cultivated which express a fusion protein which
20 comprises the specific cleavage site of presenilinase.
   4. Process according to one of claims 1 and 2, characterised in that the fusion protein comprises amyloid β.
   5. Process according to one of claims 1 and 2,
25 characterised in that the fusion protein comprises a fragment of amyloid β.
   6. Process according to one of claims 1 and 2, characterised in that the fusion protein comprises the amyloid precursor protein or a fragment thereof.
30 7. Process according to one of claims 1 and 3, characterised in that the fusion protein comprises presenilin 1 or a fragment thereof.
   8. Process according to one of claims 1 and 3, characterised in that the fusion protein comprises
35 presenilin 2 or a fragment thereof.

29

9. Process according to one of claims 1 to 8,
characterised in that the process is a high throughput
screening test.
10. Process according to one of claims 1 to 9,
5   characterised in that the quantity of reporter cleaved is
measured with luciferase, green fluorescent protein or a
derivative thereof, or secretory alkaline phosphatase or
β-galactosidase.
11. Process according to one of claims 1 to 10,
10   characterised in that the quantity of reporter cleaved is
detected indirectly.
12. Process according to one of claims 1 to 11,
characterised in that the quantity of reporter cleaved is
detected indirectly, in that a cleaved reporter domain
15   migrates into the nucleus, binds there to another
reporter domain of a reporter construct and the
expression of the reporter is activated.
13. Process according to one of claims 1 to 12,
characterised in that the quantity of cleaved reporter
20   Gal4 is detected indirectly, in that Gal4 in the nucleus
binds to the Gal4DNA binding domain of another reporter
construct and the expression of luciferase is activated.
14. Process according to one of claims 1 to 13,
characterised in that the quantity of reporter cleaved is
25   detected directly.
15. Process according to one of claims 1 to 14,
characterised in that the cells are of neuronal or glial
origin.
16. Process according to one of claims 1 to 15,
30   characterised in that the cells are H4 cells.
17. High throughput screening test for finding
substances capable of specifically inhibiting
γ-secretase, characterised in that
a) H4 cells are cultivated which endogenously exhibit
35   said γ-secretase activity and express the membrane-
associated recombinant fusion protein Aβ-KKK/Gal4,

b) these cells are incubated with a test substance,

c) the quantity of transactivator Gal4 cleaved and
binding to the Gal4-DNA binding domain of the
luciferase construct is measured by means of the level

5   of luciferase expression and

d) the value obtained is compared with the value obtained
in the absence of the test substance.

18.   Use of a process or high throughput screening test
according to one of claims 1 to 17 for finding substances

10   capable of specifically inhibiting γ-secretase.

19.   Use of a process or high throughput screening test
according to one of claims 1 to 17 for finding substances
capable of specifically inhibiting presenilinase.

20.   Substance which can be found by a process or high

15   throughput screening test according to one of claims 1 to
17, characterised in that it specifically inhibits the
proteolytic cleaving of a γ-secretase substrate.

21.   Substance which can be found by a process or high
throughput screening test according to one of claims 1 to

20   17, characterised in that it specifically inhibits the
proteolytic cleaving of a presenilinase substrate.

22.   Use of a substance according to claim 20 or claim 21
for preparing a medicament for the treatment of
neurodegenerative disorders, particularly Alzheimer's

25   disease.

23.   Pharmaceutical formulation, characterised in that it
contains a substance according to claim 20 or 21 as well
as conventional pharmaceutical carriers.

Constructs:

gal4-DNA binding domain　　　reporter gene
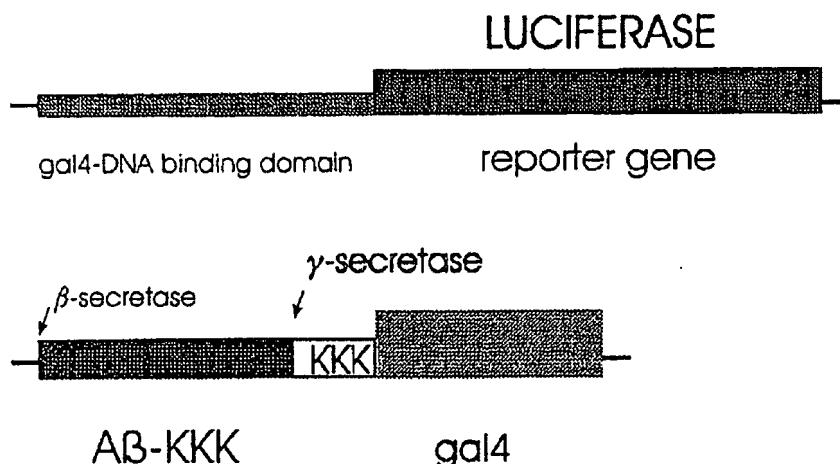
AβKKK　　　　　　　gal4

Double stable cell line:　　　Aβ-KKK/gal4
　　　　　　　　　　　　　　　　gal4dbd/Luc
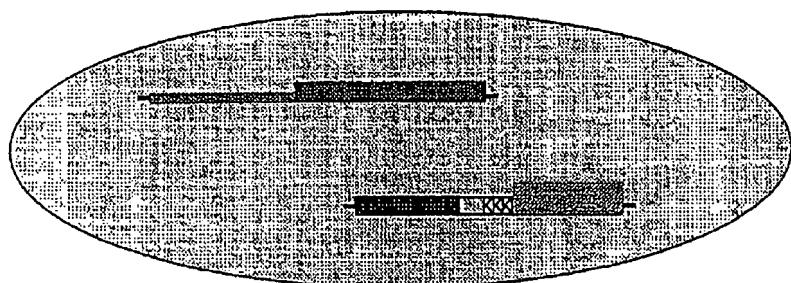
Fig. 1

Assay Design:

- grow double stable cell line on 384 well microtiter plate
- incubate with substances (overnight or for a shorter period of time)
- measurement of luciferase activity in a luminometer (5-10 sec.)


Fig. 2

Fig. 3

Fig. 4 a

double stable   stable

Aß-KKK-ER   Aß-KKK

40 59 65   40 52   u.t.   luc53

← Aß-KKK-gal4

Fig. 4 b

double stable   stable   transiently transf.

+ Luc-gal4dbd

Luciferase   Aß-KKK-ER   Aß-KKK

40 59 65   40 52   u.t. luc53   Sol. Gal4 Aß-KKK Aß-KKK-ER

← Luciferase

Fig. 4 c

Fig. 4 d

Fig. 5

Fig. 6 a

Fig. 6 b

Fig. 7 a

Effect of PS (Asp) Mutations in HTS Cell Line Expressing the
Aß-KKK Substrate without the ER Retention Signal

Fig. 7 b

Effects of PS (Asp) Mutations in HTS Cell Lines Expressing
the Aß-KKK-ER Substrate Carrying the
ER Retention Signal

Fig. 7 c

Fig. 8 a

Fig. 8 b

Fig. 9

Compound C in the gamma-Secretasetest
(clone H4/Aß-KKK-ERret.59, 65, 40; H4/Aß-KKK.52)

Fig. 10